ANTIGENS OF ARBACIA SPERM EXTRACTS[1]

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The initial steps in fertilization appear to involve interactions of the sperm and egg surfaces at the molecular level (see Tyler, 1948, 1949; Metz, 1957a, 1957b). Most of the present information concerning such interaction has been obtained from studies of egg and sperm extracts. Among sperm extracts those with action on eggs have commanded most interest. In the sea urchin and certain other forms, extracts prepared by a variety of methods precipitate the egg jelly layer, agglutinate eggs and neutralize the sperm agglutinating action of the fertilizin obtained from eggs. These effects of the extracts may result from the action of the sperm-surface receptor substance, antifertilizin, with which fertilizin combines in the sperm agglutination reaction (*e.g.* Tyler, 1948; Metz, 1957b). Whether or not these effects are to be identified with antifertilizin, absorption experiments have shown that such extracts contain some antigens in common with those of the sperm surface (Köhler and Metz, 1959a, 1959b, 1960). Further examination to reveal a more complete spectrum of antigens in such extracts seemed desirable. Accordingly, in the present investigation, *Arbacia* sperm extracts were examined for antigens by means of agar diffusion and immunoelectrophoretic techniques. The study revealed a maximum of four antigens in extracts prepared by freeze-thawing sperm.

Material and Methods

Arbacia punctulata from the vicinity of the Florida State University Marine Laboratory, Alligator Point, Florida, and from Woods Hole, Massachusetts, were used in the study. Semen was usually obtained from the animals by electrical stimulation (Harvey, 1956). The spermatozoa were separated from the seminal plasma by centrifugation (approximately $3000 \times g$; 20 minutes) at 4° C. The packed sperm were resuspended once in sea water and settled again by low speed centrifugation. The final supernatants following such washing regularly failed to give precipitation bands when diffused against anti-sperm serum. Standard sperm suspensions were prepared by diluting the packed sperm with three volumes of sea water.

Sperm extracts were prepared from such suspensions of washed sperm by a variety of methods. These included the established methods for preparing agents

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which act upon the egg jelly layer, namely, heating sperm to 100° C. (Frank, 1939) and freeze-thawing sperm (Tyler, 1939). The latter extracts are called "frozen-thawed extracts" below. Other methods are described with the individual experiments.

Antisera were prepared by injecting rabbits with sperm (25% washed sperm in sea water). The immunizing antigens were administered through intravenous, intraperitoneal and subscapular injections. In the last instance the antigen was injected as an emulsion in Freund's adjuvant (Difco). Several anti-*Arbacia* sera were examined. With the exceptions noted in the text the experiments reported here were performed with serum from the hyper-immune rabbit "#33." This rabbit received three injections of antigen in Freund's adjuvant (Difco) over a period of five months and was bled two weeks subsequent to the final injection. The immune serum obtained regularly agglutinated sperm to dilutions of 2^{-8} to 2^{-10}. No sera were pooled.

Agar diffusion and electrophoresis. Agar diffusion experiments using the technique of Ouchterlony (1948) were performed in 2% agar containing Merthiolate (0.01%) as a preservative. The reaction plates were incubated at room temperature for several weeks. Immunoelectrophoretic analysis (Wunderly, 1957) was performed using 2% agar blocks prepared in 0.05 ionic strength veronal buffer, pH 8.5 and containing 0.01% Merthiolate. Wells in the agar block were filled with antigen prepared as follows: after dialysis against 0.05 ionic strength veronal, the antigen was heated to 45° C. and mixed with an equal volume of melted (45° C.) 4% agar, also in veronal buffer. The mixture was then pipetted into the antigen wells of the agar slab and allowed to solidify. Agar slabs measuring approximately 20 × 6.05 cm. with antigen wells of 0.3–0.4 ml. capacity were used in these experiments. To achieve electrophoretic migration the preparations were subjected to a current of 25 ma for about six hours.

To improve the resolution of precipitin bands the agar blocks were fixed in 2% acetic acid, stained with Amidoschwarz (0.1% in acetate buffer, pH 4.0 solution) and destained in methanol-water-acetic acid (45:45:10).

Results

Agar diffusion precipitin tests were performed on extracts prepared by freeze-thawing washed *Arbacia* sperm and subsequently centrifuging the extracts in a clinical centrifuge (approximately 3000 × *g*). When diffused against anti-whole sperm serum such extracts yielded a maximum of four precipitin bands. Proceeding from the antigen to the antibody well in the agar plate, these four bands are designated a, b, c, and d. As seen in Figure 1, some variation in band number was found in repeated tests (*e.g.* two bands in Figures 1a5, 1b2, 1b3; three bands in 1b4, 1c6; four bands in 1c1, 1d3). Antisera other than #33 gave one to two bands. These differences in tests with different "frozen-thawed extracts" using serum #33 are attributed to differences in antigen concentration. However, the possibility of qualitative differences has not been eliminated. Differences in the band number using other antisera may reflect differences in concentration of antibody as well as antigen in the tests. In view of the fact that the sperm were washed sufficiently before extraction to remove seminal plasma antigens, the pre-

cipitin bands in the extracts must have arisen from antigens extracted from the sperm cells.

In this connection it is of interest that undiluted seminal plasma forms three bands when diffused against anti-whole sperm serum (Fig. 1a4, 1b1). Two of these seminal plasma bands join but do not cross two of the "frozen-thawed extract" bands. It appears, then, that the seminal plasma shares at least two antigens with the extract. In fact these seminal plasma antigens may have diffused from the

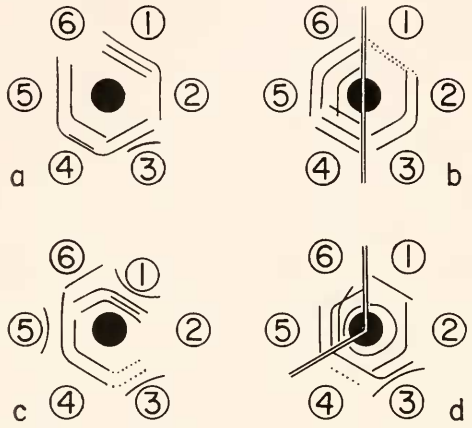

FIGURE 1. Ouchterlony agar diffusion tests. The center wells of all plates contain anti-*Arbacia* sperm serum No. 33. All surrounding wells were filled with extract and fluid of *Arbacia punctulata*. Each well received 0.5 ml. of the sample: (a) (1) supernatant over frozen-thawed sperm after centrifugation at 26,000 × *g* for 20 minutes; (2) residue of No. (1), resuspended in sea water and centrifuged at low speed; (3) supernatant over Mickle-disintegrated sperm; (4) seminal plasma; (5) "frozen-thawed extract" of sperm, low speed centrifuged; (6) body fluid; (b) (1) seminal plasma; (2) "frozen-thawed extract" of whole sperm, low speed centrifugation; (3) "frozen-thawed extract" of whole sperm, low speed centrifugation; (4) "frozen-thawed extract," low speed centrifugation; (5) supernatant over Mickle-disintegrated sperm, low speed centrifugation; (6) supernatant over Mickle-disintegrated sperm, low speed centrifugation; (c) (1) "frozen-thawed extract," low speed centrifugation; (2) basic protein, pH 0.9 extract; (3) supernatant over washed sperm after standing (aging), low speed centrifugation; (4) supernatant over citric acid-extracted sperm, low speed centrifugation; (5) supernatant over urea-treated sperm; (6) frozen-thawed extract, low speed centrifugation; (d) (1) isolated heads, "frozen-thawed extract," low speed centrifugation; (2) isolated tails, "frozen-thawed extract," low speed centrifugation; (3) whole sperm, "frozen-thawed extract," low speed centrifugation; (4) isolated heads, "frozen-thawed extract," low speed centrifugation; (5) acid extract (pH 3) of sperm, low speed centrifugation; (6) acid extract (pH 1.9) of sperm, low speed centrifugation.

sperm. However, the one band that has been clearly demonstrated in the supernatant of aging sperm is band *a* of "frozen-thawed extract" (Fig. 1c3). It should be noted that the three bands just described do not constitute the complete antigenic spectrum of *Arbacia* seminal plasma. As seen in Figure 2d, immunoelectrophoresis resolved seven bands in this material. Three of these correspond in position to three precipitin bands in the sperm extract (Fig. 2c).

It should be clear from these results that extracts prepared by freeze-thawing

sperm are not solutions of a single macromolecule. On the assumption that each precipitin band represents a single antigen, the extracts can contain up to four different antigens. Extracts of sperm prepared by other procedures also contained antigens. As seen in Figure 1d5, extraction of *Arbacia* sperm at pH 3 yielded a preparation which produced four precipitin bands. Evidently two of these antigens are labile to or are insoluble in stronger acids for, as seen in Figure 1d6, an aliquot of the same sperm suspension extracted at pH 1.9 yielded but two precipitin bands. Extraction at even lower pH (pH 0.9) yielded preparations that failed to form precipitin bands. The extracts prepared by extracting *Arbacia* sperm at pH 3 failed to precipitate egg jellies.

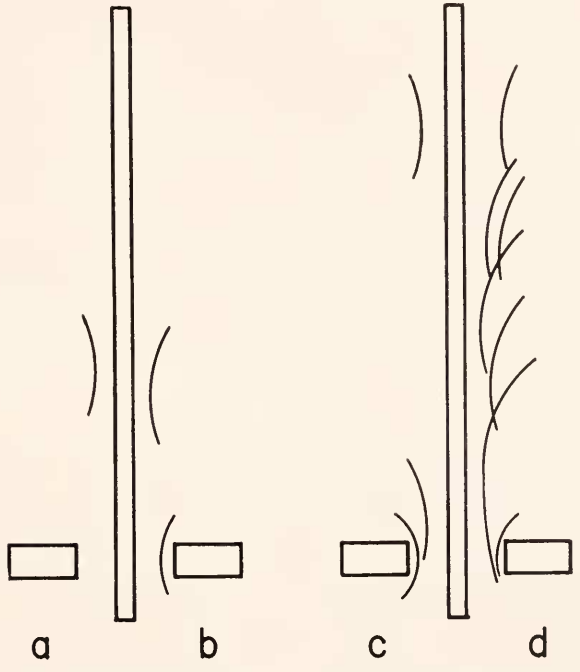

FIGURE 2. Immunoelectrophoresis in agar of various fluids from *Arbacia*. The central channel contains anti-*Arbacia* sperm serum No. 33, lateral channels contain preinjection serum No. 33. (a) Heated sperm extract; (b) "frozen-thawed extract," low speed centrifugation; (c) "frozen-thawed extract," low speed centrifugation; (d) seminal plasma.

Likewise, extracts prepared by treatment of sperm in the Mickle disintegrator (20 minutes, 20° C.) yielded three precipitin bands (Fig. 1b5 and 1b6). These have not been homologized with the antigens of "frozen-thawed extract." However, two (Fig. 1b4 and 1b5) of the bands correspond in position to two of the bands from "frozen-thawed extract." The third band crosses one of the bands of the "frozen-thawed extract" (probably the *d* band) and may represent an antigen not present in "frozen-thawed extract." Other extracting agents used were 1/18 *M* Na-citrate and 4 *M* urea. As seen in Figures 1c4 and 1c5, citrate extract pro-

duced two bands and urea extract three. The two citrate extract bands join two of the three urea extract bands. The third band of the urea extract is probably identical with the *a* band of "frozen-thawed extract."

As might be expected, extracts prepared by heating (100° C.) *Arbacia* sperm for one to five minutes do not have the full complement of soluble antigens. Such extracts at most yield a single band when diffused against anti-whole sperm serum. This antigen is evidently present in low concentration in extracts prepared by heating 25% sperm suspensions, for most such preparations fail to produce any precipitin bands in Ouchterlony or immunoelectrophoretic tests (*e.g.* Fig. 3a3). The band that does appear corresponds in position to one of the immunoelectrophoresis bands of "frozen-thawed extract" (see Figure 2 and below).

As seen above, extracts of whole sperm can contain at least four precipitating antigens. As in the case of the sperm agglutination antigens (Köhler and Metz, 1960), it seemed of interest to attempt to determine if these are present in both sperm heads and tails. Accordingly, sperm were broken into heads and tails by Mickle disintegration, these structures were separated by differential centrifugation and finally extracted by freeze-thawing (for details, see Köhler and Metz, 1960). When examined for precipitating antigens by agar diffusion, the frozen-thawed whole sperm extract produced four bands (Fig. 1d3). Head and tail extracts, adjusted to corresponding concentration, each produced two bands. One of these was common to both extracts and joined the *d* band of whole sperm extract. The second band in the head and tail extracts also joined bands produced by whole sperm extracts. However, these bands were not (Fig. 1d4) identical. The second band from the head extract joined the *b* band of whole sperm extract whereas the second band of tail extract joined the *c* band of the whole sperm extract (Fig. 1d2).

From the foregoing, it appears that both head and tail extracts lack band *a* of whole sperm extract. The absence of band *a* in the head and tail extracts is attributed to loss of this readily extracted antigen in the isolation process. Band *b* is present in extracts of isolated heads, but not tails; band *c* is present in tails but not heads and band *d* is present in both head and tail extracts.

Immunoelectrophoresis. In attempts to further resolve the antigenic composition of sperm extracts and seminal plasma these were subjected to immunoelectrophoresis in agar blocks (2% agar in 0.05 ionic strength veronal, pH 8.6). This method resolved three precipitin arcs (in three separate experiments) in frozen-thawed sperm extracts. As seen in Figure 2, b and c, one of these moved rapidly toward the anode, a second moved with intermediate speed and the third did not move appreciably. In comparative studies seminal plasma produced seven definite arcs (Fig. 2d). Three of these corresponded in position to the three arcs of the frozen-thawed sperm extract. Heat-extracted sperm (100° C., one minute) never produced more than a single arc (Fig. 2a). This corresponded in position to the fastest migrating antigen of unheated extract and seminal plasma.

Experiments with extracts centrifuged at high speed. In the studies presented above (Figs. 1 and 2), the extracts of frozen-thawed sperm were centrifuged at low speed (*e.g.* $3000 \times g$) only. Upon high speed centrifugation (*e.g.* $26{,}000 \times g$) of such extracts a pink, semi-gelatinous pellet is obtained and much, if not all of the egg jelly precipitating activity of the extracts is associated with this sedi-

mentable material (Köhler and Metz, 1959b). Therefore, a comparative study of extracts before and after such high speed centrifugation was undertaken. This showed that three of the bands (b, c, d) remained in the supernatant of "frozen-thawed extract" after centrifugation at 26,000 × *g* (Fig. 3a1 and 3a4). The *a* band was absent from such preparations. The pink pellet obtained after the high speed centrifugation was homogenized in a small amount of sea water, and gave only the *a* band when diffused against anti-sperm serum (Fig. 3a2 and 3a6). This result suggests that the *a* band (e.g. in Fig. 1a3, 1c1, 1c3 and 1d3) represents antigenic material, that is readily sedimented by high speed centrifugation.

In comparative studies with extracts prepared by other means and centrifuged at high speed, frozen-thawed and acid extracts (pH 3.2) showed one band (probably the *c* band) in common (Fig. 3a5). In a second experiment (Fig. 3c and 3d) only two bands were resolved in the high speed supernatant of "frozen-thawed extract." One of these (probably the *b* band) is common to the high speed supernatants of seminal plasma (Fig. 3c3) and heat extract (Fig. 3c6) and the low speed sediment of the original "frozen-thawed extract." Histone prepared by pH 0.8 extraction of sperm and nucleoprotein prepared according to Mirsky and Pollister (1942) gave no precipitation bands with the antiserum after high speed centrifugation (Fig. 3d). This is not surprising since histone and nucleoprotein are generally found to be poor antigens (Cushing and Campbell, 1957).

The supernatant obtained after high speed centrifugation of "frozen-thawed extracts" was also compared with extracts obtained by aging sperm (48 hours, in sea water), extraction with 4 *M* urea and with 1/18 *M* citrate for 48 hours (Fig. 3b). One band, possibly the *c* band, is common to all of these extracts.

Discussion

The results reported here show that extracts of frozen-thawed *Arbacia* sperm can yield four distinct precipitation bands when diffused against anti-whole sperm serum. These four bands are interpreted as four separate antigens. Detection of all of these antigens in agar diffusion precipitin tests appears to depend upon having high titer antisera and concentrated solutions of extract. Indeed these antigens may be only slightly soluble substances. Other extraction procedures (*e.g.* heating, acid, citric acid, and urea extraction) have not clearly revealed additional antigens. Likewise immunoelectrophoretic analysis revealed three antigens in frozen-thawed extracts. It appears, then, that only a few sea water-soluble antigens are obtained from *Arbacia* sperm in appreciable concentration. Possibly immunization of additional rabbits or other animals might yield an antiserum of unusual resolving power and reveal additional sperm antigens. However, the experience so far suggests that but few additional antigens would be discovered. The relatively small number of antigens in sperm extracts is in sharp contrast to *Arbacia* seminal plasma and egg extracts prepared by freeze-thawing. Parallel immunoelectrophoretic studies using anti-*Arbacia* egg sera and frozen-thawed extracts of *Arbacia* eggs readily resolved nine antigens. Likewise Perlmann (1953) found at least ten precipitin bands in agar diffusion tests using 0.15 *M* NaCl extracts of *Paracentrotus lividus* eggs. In preliminary tests no cross precipitin reactions were obtained between sperm extracts and anti-egg sera or the reverse.

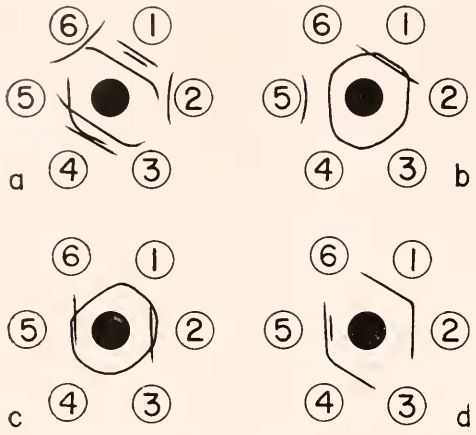

FIGURE 3. Ouchterlony agar diffusion tests. The center wells of all plates contain anti-*Arbacia* sperm serum No. 33. All surrounding wells were filled with extracts or fluids of *Arbacia punctulata*. Each well received 0.5 ml. of sample: (a) (1) "frozen-thawed extract" immediately subjected to centrifugation at 26,000 × *g*; (2) the pellet from No. 1 and No. 4, washed and resuspended; (3) heat extract, centrifuged at 26,000 × *g*; (4) "frozen-thawed extract" immediately subjected to centrifugation at 26,000 × *g*; (5) acid extract, centrifuged at 26,000 × *g*; (6) the pellet from No. 1 and No. 4, washed and resuspended. (b) (1) A very active egg jelly precipitating preparation obtained by resuspending the cake of frozen-thawed sperm followed by centrifugation at low speed; (2) 4 *M* urea extract of sperm, centrifuged at 26,000 × *g*; (3) 1/18 *M* Na-citrate extract of sperm, centrifuged at 26,000 × *g*; (4) supernatant over aged sperm centrifuged at 26,000 × *g*; (5) 4 *M* urea extract of sperm centrifuged at 26,000 × *g*; (6) 1/18 *M* Na-citrate extract of sperm, centrifuged at 26,000 × *g*. (c) (1) and (4) Supernatant over frozen-thawed sperm after high speed centrifugation; (2) and (5) the cake of frozen-thawed sperm was resuspended (same as Figure 3b1) in sea water for additional extraction (wells contain the supernatant after low speed centrifugation); (3) seminal plasma centrifuged at 26,000 × *g*; (6) heat extract, centrifuged at 26,000 × *g*. (d) (1) and (4) Supernatant over frozen-thawed sperm after high speed centrifugation; (2) and (5) the cake of frozen-thawed sperm was resuspended (same as Figure 3b1) in sea water for additional extraction (wells contain the supernatant after low speed centrifugation); (3) nucleoprotein centrifuged at 26,000 × *g*; (6) histone centrifuged at 26,000 × *g*.

Perlmann (1953), however, obtained one precipitin band when saline (0.14 *M*) extract of sperm was diffused against anti-egg serum.

The relationship, if any, of the four soluble sperm antigens to the sperm surface and to the egg jelly precipitating activity of the extracts has not been examined in detail. However, it is likely that the three antigens, *b*, *c*, and *d*, of "frozen-thawed extracts" are not related to the egg jelly precipitating activity because the latter activity is removed by high speed centrifugation whereas the *b*, *c*, *d* antigens are not sedimented by such centrifugation (Köhler and Metz, 1959b). With regard to the relationship of the soluble antigens to sperm surface antigens it should be recalled that absorption of anti-sperm serum with "frozen-thawed extracts" lowers the titer but does not completely neutralize the sperm agglutinating action of such antiserum (Köhler and Metz, 1960). This shows that the extracts contain some but not all of the sperm surface antigens. Agar diffusion experiments employing sera absorbed with whole sperm might reveal whether the soluble antigens are surface or subsurface material.

Summary

1. Extracts were prepared from *Arbacia* sperm by several procedures. These were tested for antigenic composition by diffusion against anti-*Arbacia* sperm rabbit serum on Ouchterlony plates and by means of immunoelectrophoresis in agar gel.

2. Extracts prepared by freeze-thawing the sperm followed by low speed centrifugation produced a maximum of four precipitin bands. It is concluded that such extracts contain at least four soluble antigens.

3. Seminal plasma revealed seven arcs in an immunoelectrophoretic experiment, and a maximum of three bands on Ouchterlony plates. Two such bands join bands from "frozen-thawed extracts."

4. Extracts prepared by heating sperm at 100° C. yielded at the most one band. This antigen seemed to be common to several other extracts.

5. Nucleoprotein (Mirsky and Pollister) and histone failed to form precipitin bands.

6. One of the four bands in frozen-thawed extract is associated with material sedimented at 26,000 × *g*.

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